

The Role of Human Endogenous Retrovirus (HERV) K Encoded Deoxyuridine Triphosphate
Nucleotidohydrolase in Psoriasis

Honors Research Thesis

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by

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Abstract

Psoriasis is a chronic inflammatory disease that affects 2-3% of the worldwide population. It is characterized by an epidermal hyperproliferation and an inflammatory cell infiltrate into the epidermis and dermis that results in the development of psoriatic lesions. Presently, the drugs used for treatment are generally immunosuppressive agents that either suppress the activation of immune response cells or inhibit cytokine/chemokine molecules that promote the inflammatory microenvironments characteristic of psoriasis pathophysiology. It has been reported that the target region of the psoriasis susceptibility locus (PSORS1) harbors a fragment of a human endogenous retrovirus K (HERV-K), which encodes for a deoxyuridine triphosphate nucleotidohydrolase (dUTPase). Our hypothesis is that the HERV-K dUTPase protein triggers a Th1, Th17 cytokine response by activating NF- κ B through toll-like receptor 2 (TLR2). The goal of my study was to identify the region of the HERV-K dUTPase responsible for activating TLR2. To accomplish this I constructed three specific deletion constructs (Δ 60-171; Δ 90-171; and Δ 110-171) of the consensus HERV-K dUTPase sequence via PCR amplification and cloned these DNA fragments into the pTrcHis vector for expression and subsequent purification of the recombinant proteins. The recombinant proteins were purified using cobalt affinity chromatography. The purified recombinant proteins will be used to determine structural characteristics of the recombinant protein and to determine whether these mutant proteins activate a NF- κ B reporter gene in human embryonic kidney (HEK-293) cells expressing TLR2. We believe that successful completion of our studies will enable our research group to pursue development of a vaccine to generate antibodies that specifically target our identified region and thus provide a novel alternative treatment option for psoriasis patients.

Introduction

Psoriasis is an inheritable chronic inflammatory disease of the skin affecting 2-3% of the world's population. It is characterized by an epidermal hyperproliferation and an inflammatory cell infiltrate into the epidermis and dermis that typically results in the development of psoriatic lesions. Previous epidemiological and genetic studies have indicated that the establishment of the disease can be attributed to a variety of risk genes, environmental factors, excessive stress, trauma, and a variety of innate and adaptive immune cell interactions which are responsible for the production and secretion of various cytokines and chemokines commonly attributed to psoriasis symptomology (Lowes et al., 2007; Nickoloff 2007; Nickoloff et al., 2007; Nestle et al., 2009).

The psoriasis susceptibility locus 1 (PSOR1), located in the major histocompatibility complex on chromosome 6p21, has been identified as the strongest genetic determinant of psoriasis, accounting for 35-50% of the heritability of the disease. Recent haplotype sharing analysis has revealed that the PSOR1 locus contains fragments of the human endogenous retrovirus K (HERV) genome, specifically segments of the C-terminal *gag* gene and the N-terminal *pro* gene, which harbors the region encoding the deoxyuridine triphosphate nucleotidohydrolase (dUTPase) (Foerster et al., 2005).

HERVs are known to constitute approximately 7-8% of the human genome and are the result of ancient exogenous retroviruses infecting human germ-cell lines millions of years ago (Lower et al., 1995). It is thought that these endogenous proviruses may have conferred protection to their hosts from superinfection by pathogenic exogenous counterparts and thus provides explanation for their maintenance within the human genome (Lower et al., 1995).

Endogenous retrovirus genomes have undergone a variety of mutational events since their incorporation into the human genome and have thus lost their ability to retrotranspose, replicate, and infect additional cells. As such, HERV genomes no longer encode for completely intact virions, but rather a variety of proteins that have generally undergone mutational development over time. The fact that these HERV open reading frames are still intact indicates potential protein functionality within the host (Lower et al., 1995). However, there are several reports that have linked both human endogenous and exogenous retroviruses to the development of autoimmune disorders including psoriasis (Bannert and Kurth, 2004; Colmegna and Garry, 2006; Guilhou and Moles, 2008). HERV-K is generally recognized as the most biologically active endogenous retrovirus and until now has been largely associated with its role in development of seminoma, melanoma, and insulin-dependent diabetes mellitus (Voisset et al., 2008). The recent link between the PSOR1 HERV-K encoded dUTPase and psoriasis symptomology is further supported by detection of HERV-K encoded dUTPase RNA in peripheral blood mononuclear cells (PBMCs) of psoriasis patients using RT-PCR analysis (Foerster et al., 2005).

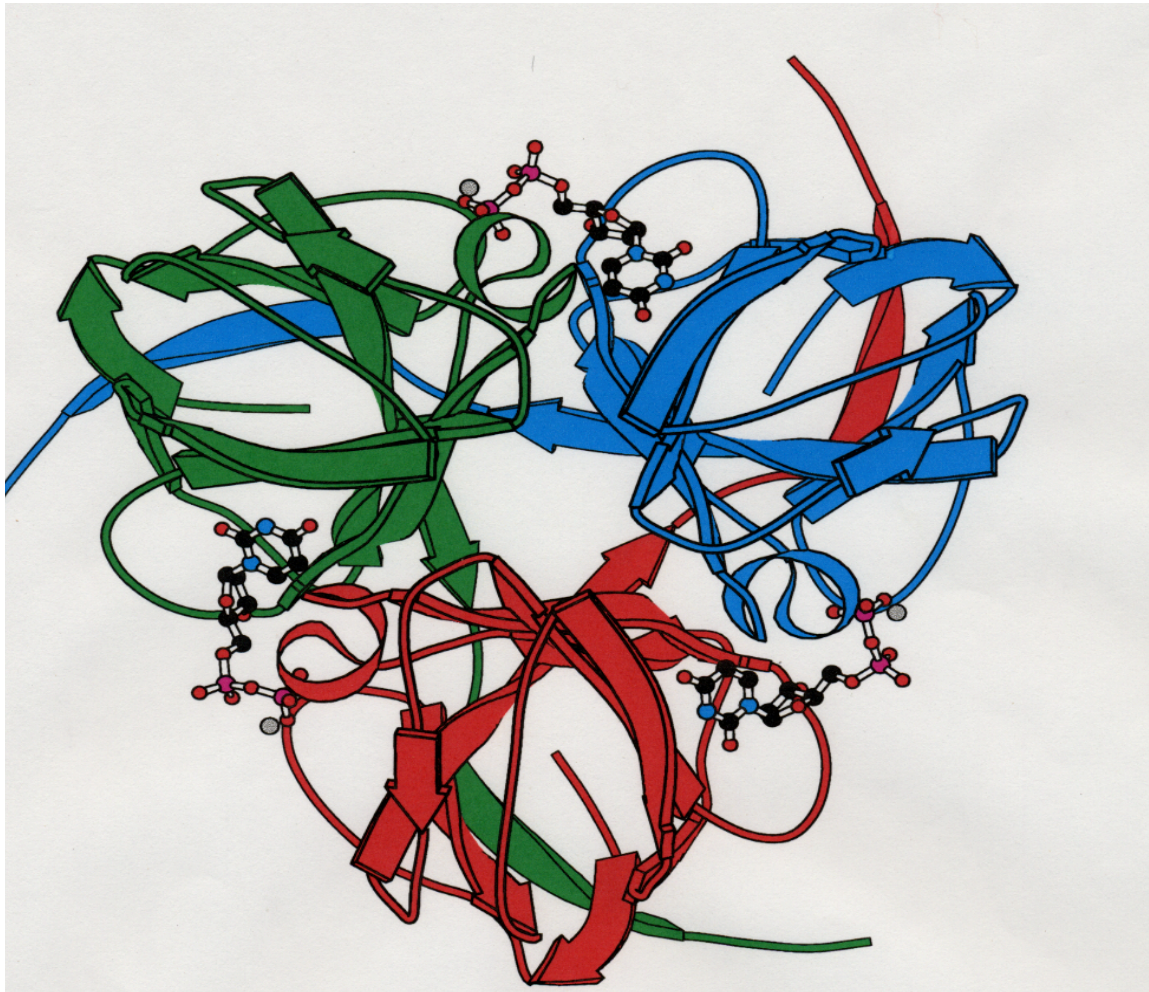
HERV-K is classified as an endogenous beta retrovirus. As a result of a slippery sequence (poly U region followed by a hairpin loop) in the beta retrovirus genome, ribosomal frameshifting occurs which enables the ribosome to transcribe both the *gag* and *pro* genes consecutively, leading to formation of a Gag-Pro polyprotein. Cleavage of the polyprotein results in the formation of a NC-dUTPase fusion protein, which is not subject to the proteolytic activity separating the nucleocapsid from the dUTPase common to all other retroviruses (Nemeth-Pongracz et al., 2006).

dUTPase proteins, metalloenzymes found in all living organisms, hydrolytically cleave dUTP into dUMP and pyrophosphate, thus preventing DNA polymerase from incorporating

dUTP into DNA during DNA replication (McGeoch, 1990; Mayer and Meese, 2003). Homotrimeric (Figure 1) and monomeric dUTPases contain five highly conserved domains that allow for enzymatic activity. A previous study on the Mason-Pfizer Monkey Virus, a beta exogenous retrovirus, has indicated that both domains II and V are essential for enzymatic activity in addition to revealing the inherent flexibility of domain V (Nemeth-Pongracz et al., 2006). Enzyme kinetic studies have revealed that for beta exogenous retroviruses, the NC-dUTPase fusion protein is fully functional, as the dUTPase is not sterically hindered by the presence of the fused nucleocapsid protein (Nemeth-Pongracz et al., 2006). Although the HERV-K dUTPase contains the first four conserved domains, its fifth domain is the least conserved (Figure 2). The HERV-K dUTPase however has been shown to possess zero enzymatic activity (Harris et al., 2007; Ariza and William, 2011).

While all human herpes viruses form monomeric dUTPases that likely evolved from human dUTPases via tandem repeat duplication, the human, beta (endogenous and exogenous) retroviral, and most bacterial dUTPases form homotrimeric quaternary structures. The three active sites of homotrimeric dUTPases are formed via the interaction of domains I, II, and IV from one monomer with domain III from an adjacent monomer. Arm swapping occurs between each monomer's characteristically flexible domain V, which acts as a movable arm presenting dUTP to the active site (Nemeth-Pongracz et al., 2006) (Figure 1). Conversely, a monomeric dUTPase has rearranged the five conserved domains from a numerically consecutive order as that found in a homotrimeric dUTPase to an order consisting of domain III, I, II, IV, and V. With this rearrangement, the N-terminal domain III folds back onto domains I, II, and IV to form the active site, while the C-terminal domain V again works as a flexible arm bringing the dUTP substrate to the active site.

Figure 1.



The first evidence of a dUTPase possessing immunomodulatory activity was demonstrated by Drs. Ariza and Williams' group (Glaser et al., 2006, Ariza et al., 2009) using the Epstein-Barr Virus (EBV) encoded dUTPase. EBV is a gamma herpes virus and thus its dUTPase has a monomeric quaternary structure. PBMCs, induced to proliferate via anti-CD3-mAb, were treated with purified EBV-encoded dUTPase, which resulted in the arrest of T-cell replication, while unstimulated PBMCs treated with purified EBV-encoded dUTPase had upregulated proinflammatory cytokine expression including TNF- α , IL-1 β , and IL-8 (Glaser et al., 2006). Furthermore, the EBV-encoded dUTPase was demonstrated to induce comparable

immune dysregulation in human monocyte-derived macrophages (hMDM)-CD14⁺ via activation of the NF- κ B transcription factor (Ariza et al., 2009). A subsequent study used a luciferase reporter assay with a HEK-293 stable cell lines expressing TLR2 to demonstrate that purified EBV-encoded dUTPase activated NF- κ B through TLR2 and that this signaling cascade was dependent on the MyD88 adaptor signaling molecule (Ariza et al., 2009).

The discovery of EBV-encoded dUTPase immunomodulatory activity and identification of a HERV-K encoded dUTPase within the PSOR1 locus prompted our research group to develop studies to determine if the HERV-K encoded dUTPase possesses comparable immunomodulatory functionality in the context of psoriasis. Because psoriasis is characterized by chronic epidermal hyperproliferation and inflammation, it is logical to hypothesize that the HERV-K encoded dUTPase could function with a mechanism similar to the EBV-encoded dUTPase in initiating the production of proinflammatory cytokines. Initial studies conducted by Drs. Ariza and Williams revealed that the HERV-K dUTPase was able to induce activation of NF- κ B through TLR2 and that treatment of human primary cells with the HERV-K dUTPase resulted in the Th1, Th17 cytokine response associated with the induction of psoriasis symptomology (Ariza et al., 2011 In Press Journal of Investigative Dermatology). Specifically, TNF- α , IL-23, IL-17, IL-9, IL12p40, and INF- γ , which are associated with the production of psoriatic plaques, and CCL20 and RANTES, which are chemokines involved in leukocyte recruitment to the skin, were produced by the human primary cells in these studies (Ariza et al., 2011 In Press Journal of Investigative Dermatology). Not only did the wild-type (WT) HERV-K encoded dUTPase reveal immunomodulatory activity, but C-terminal deletions from the WT dUTPase produced enhanced activation of NF- κ B through TLR2. Specifically, our lab developed the mutant 16 HERV-K dUTPase, lacking the C-terminal 30 amino acids (Δ 141-171),

which was shown to increase NF- κ B activation by 31.5 fold. This result indicated that the region between amino acid 141 and 171 could be important in the regulation of NF- κ B activation through TLR2.

Various drugs that are administered either topically or systemically, depending upon the severity of the disease, are used to treat patients with psoriasis (Lowes et al., 2007). These agents are generally immune modulators, which suppress the activation of cells involved in the immune response and/or inhibit the action of key cytokines reported to contribute to the inflammatory reaction that occurs in psoriasis. While these agents can be effective, they do not target the event(s) that trigger the cytokine cascade associated with psoriasis, but rather the immune response. In doing so, these drugs increase the risk of patients developing infections by various microorganisms. Our strategy bypasses these negative side effects by targeting the source of psoriasis symptoms, which we believe to be the interaction of the HERV-K dUTPase with TLR2. Based upon our previous studies, our working hypothesis is that mutations in the HERV-K dUTPase gene can increase TLR2 interaction and thus contribute to the initiation of psoriasis. The primary goal of my thesis project was to specifically determine the amino acid region of the HERV-K dUTPase responsible for activating TLR2. To achieve this task, I constructed three C-terminal deletion constructs from the consensus HERV-K dUTPase sequence, which I termed mutant 60 (Δ 60-171), mutant 90 (Δ 90-171), and mutant 110 (Δ 110-171). Because both the WT and mutant 16 HERV-K dUTPase form a homotrimer, as previously demonstrated by gel filtration assays, it was also important to determine if the C-terminal deletions resulted in monomeric proteins and whether or not the structural difference played a role in the activation of TLR2.

Materials and Methods

Competent Cells

An *E. coli* (BL21(DES)pLysS) colony was selected to start an overnight culture in LB media in the absence of antibiotics. A sample of the overnight culture (1 mL) was transferred to a flask containing LB media (100 mL) and incubated at 37°C until an OD600 of 0.5 was obtained. The flask was chilled on ice (20 min) and the cells were harvested via centrifugation (4000 rpm, 15 min, 4°C). The pellet was resuspended in resuspension buffer (85% LB medium, 10% PEG (MW 8000), 5% DMSO, 50 mM MgCl₂), aliquoted, and stored at -70°C.

Generation of Deletion Constructs

PCR amplification of Del 60 (Δ 60-171), Del 90 (Δ 90-171), and Del 110 (Δ 110-171) was performed on the consensus HERV-K dUTPase sequence using primers found in Figure 3B and PCR supermix (Invitrogen). The following PCR conditions were used: Denaturation at 94°C for 4 min (1 cycle), followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and one cycle at 72°C for 10 min. PCR products were analyzed by gel electrophoresis as they were run on a 1% agarose gel in TAE buffer and subsequently extracted from the gel using the QIAquick (QIAGEN) Gel Extraction Kit according to the manufacturer's instructions.

Cloning

The PCR amplified DNA fragments were cloned into pTrcHis plasmids using the Invitrogen pTrcHis Topo TA Cloning Kit according to the manufacturer's instructions and were used to transform BL21(DES)pLysS competent cells (figure 4). Briefly, plasmids were added to

the BL21(DES)pLysS competent cells and incubated on ice for 30 min. The cells were heat shocked for 2 min at 42°C and immediately transferred to ice. Room temperature SOC media (1 mL) was added to each cell mixture and incubated at 37°C for 2 hours while shaking. The transformed cell cultures were diluted four-fold with SOC media and spread (25 µL) onto separate petri dishes with LB media (50 µg/mL ampicillin, 0.5% glucose) and incubated overnight at 37°C. Colonies were selected from each transformation reaction and grown overnight in LB broth (50 µg/mL ampicillin, 0.5% glucose) at 37°C. Plasmid purification of the overnight cultures was performed using the Qiagen DNA Extraction Kit according to the manufacturer's instructions. Extracted DNA was subjected to PCR amplification using the previously mentioned conditions, screened on a 1% agarose gel to assess purity, and was sent for sequencing at GENEWIZ to determine if the amplified DNA fragment's orientation within the plasmid was correct.

Gene Expression and Purification

The Del 60, Del 90, and Del 110 recombinant BL21 *E. coli* mutants were each grown in LB media (6L, 100 µg/mL ampicillin, 100 µg/mL chloramphenicol) at 37°C for 2.5 hours. Protein expression was induced with IPTG (1 mM/mL) and the cultures were allowed to incubate for an additional 2 hours at 37°C. The bacteria were harvested from 6 L of media via low speed centrifugation and the bacterial pellet was resuspended in 50 mL equilibration buffer (60 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole; pH 7.4). The resuspended homogenate was centrifuged (10000 RPM, 30 min, 4°C) and the resulting supernatant was applied to Thermo Scientific HisPur Cobalt Spin Columns (3 mL) (Pierce, Rockford, IL). Recombinant proteins were eluted with 6 mL of elution buffer per column (50 mM sodium phosphate, 300 mM NaCl,

150 mM imidazole; pH 7.4). Protein concentration of each fraction was determined via spectrophotometry and fractions containing the highest concentration were combined and concentrated using Centriprep-10 centrifugal filter units (Amicon, Beverly, MA).

Protein Analysis

Polyacrylamide gel electrophoresis was performed with the Laemmli SDS PAGE system. The proteins were diluted 1:4 with sample buffer (97 mM Tris-HCL pH 6.8, 15.5% w/v glycerol, 3.1% SDS, 40 mM BME, 0.0025% bromophenol blue. The samples were then loaded on a polyacrylamide separating gel (37.5%T, 1%C) using the Bio-Rad mini gel system and run for 1-1.5 hrs at 150V with running buffer (25 mM Tris, 320 mM glycine 1% SDS, pH 6.8). Polyacrylamide gel electrophoresis was also performed with a native β -alanine gel system. The samples were loaded on a polyacrylamide separating gel (30%T, 2.7%C) using the Bio-Rad mini gel system and run for 1-1.5 hrs at 150V with running buffer (80 mM β -alanine, 40 mM Acetic acid, pH 4.4).

Results and Discussion

The successfully created deletion constructs are depicted in the amino acid sequence, with each highlighted region representing the deleted amino acids corresponding to a particular mutant (Figure 2). The pink region corresponds to Δ 141-171, mutant 16; yellow region corresponds to Δ 110-171, mutant 110; blue region corresponds to Δ 90-171, mutant 90; Green region corresponds to Δ 60-171, mutant 60. Domains I, II, III, IV, and V are underlined and in bold font. They are depicted in consecutive order from the N-terminus.

Figure 2.

KWATIVGKRA KGPASGPTTN WGIPNSAICS SGFSGTTTPT VPSVSGNKPV
TTIQQLSLTT SGSAAVDLCT IQAVSLLPGE PPQKIPTGVY GPLPEGTVGL
ILGRSSLNLK GVQIHTSVVD SDYKGEIQLV ISSIPWSAS PRDRIAQLLL LPYIKGGNSE
IKRIGGLVST D

The consensus HERV-K sequence was used for PCR amplification of the target regions to produce the Del 60, Del 90, and Del 110 deletion constructs (Figure 3A). The 5' and 3' primers utilized in the PCR reactions are depicted in Figure 3B.

Figure 3A

ggccccacaacaaactggggcattcccaattcagccctttgttctcaagggtttcagggaacaacccccactgtcccaagtacctcag
ggaataagccagttaccacaataacaattgtccccaccacaagcaacagtgcagcagtagatttatgtactatacaagcagctgtctgc
ttccaggggaacccccacaaaaatccccacaggggtatacggcccgctgcctgaggggactgtaggactgtcttgggaagatcaagtc
taaatccaaaggaggtcaaattcatactggtgtggttgattcagactataaagggtgaaattcaattgattattagctcttcaattcctgcagtc
cagtcaggagacaggatttctcaattattactcttgacttacattaagggttgaaatagtgagataaaaagaacaggaggatttggagcac
ggatctgacaggaaaggctgcatttgggcaagt

Figure 3B.

HERV-K 5' Primer

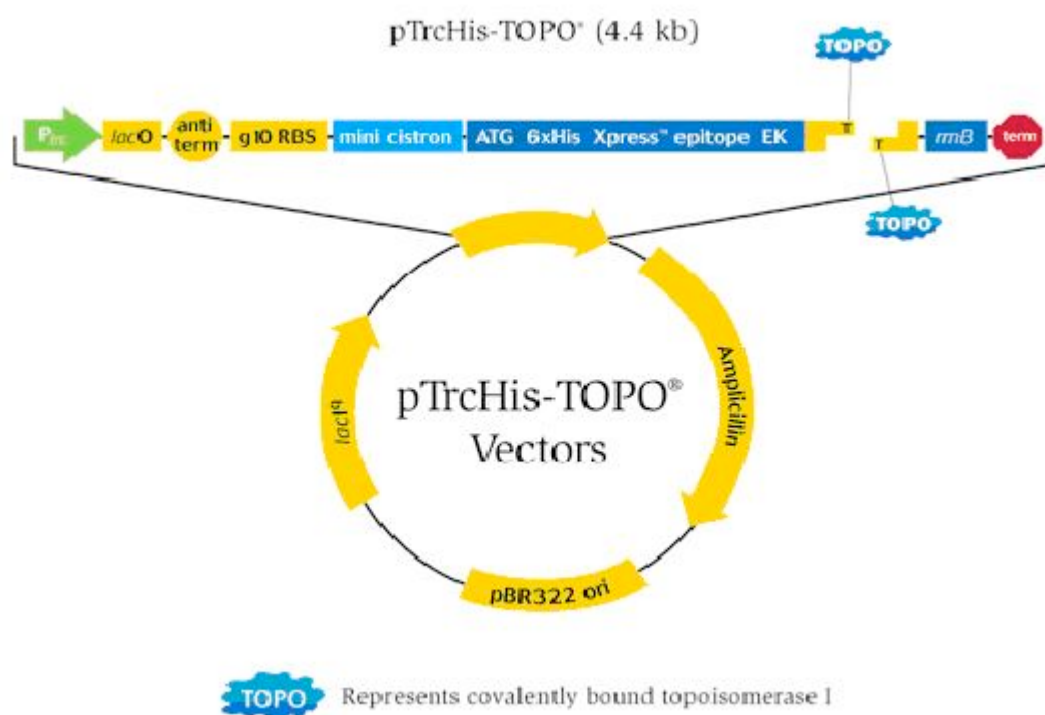
5'- F: GGATCCAAATGGGCAACCATTGTCGGGAAAC

HERV-K 3' Primers

1. Δ110: 5'- GAATTCTCACCTTTTAGATTAGACTTGATCTT-3'
2. RC16Δ90: 5'- GAATTCTCATATACCCCTGTAGGGATTTTT-3'
3. RC16Δ60: 5'- GAATTCTCAGTGGTGAGGGACAATTGTTGTATT-3'

Amplified deletion constructs were cloned into the pTrcHis Topo TA cloning vector (Figure 4). Presence of the 6 histidine residues adjacent to the multiple cloning site allowed for expressed recombinant proteins to have a built in histidine tag that permitted purification via affinity chromatography with the HisPur Cobalt Affinity Columns.

Figure 4.



Recombinant protein purity and size was determined via electrophoresis with the Laemmli SDS PAGE system. The SDS gels revealed a single band at the appropriate molecular weight as determined by sequence analysis (data not shown).

Future Studies

Luciferase assays using HEK-293 stable cell lines expressing TLR2 and an NF- κ B reporter gene have been performed. Analysis of the data is underway and a lack of NF- κ B activation should indicate the ability of mutant recombinant proteins to activate TLR2. Once this process is completed, the specific amino acid region responsible for binding TLR2 and subsequently activating NF- κ B will be determined. Furthermore, collaboration with crystallographer, Dr. Ganesaratriam Balendra, University of Akron, will allow for the determination of the crystal structure of the mutant protein interacting with TLR2. With this additional data, our research group will pursue fine point mutational development to identify the specific amino acid that is binding to TLR2. Identification of this residue will enable our research group to pursue development of a vaccine to generate antibodies that specifically target this region and thereby inhibit TLR2 activation. It is our belief that successful completion of our studies, and future drug development based upon our findings, will lead to production of a novel alternative treatment option for psoriasis patients.

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